Cell PRESS
Chemistry & Biology
Article

A Chemical Biology Strategy to Analyze Rheostat-like Protein Kinase-Dependent Regulation

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http://dx.doi.org/10.1016/j.chembiol.2013.01.003

SUMMARY

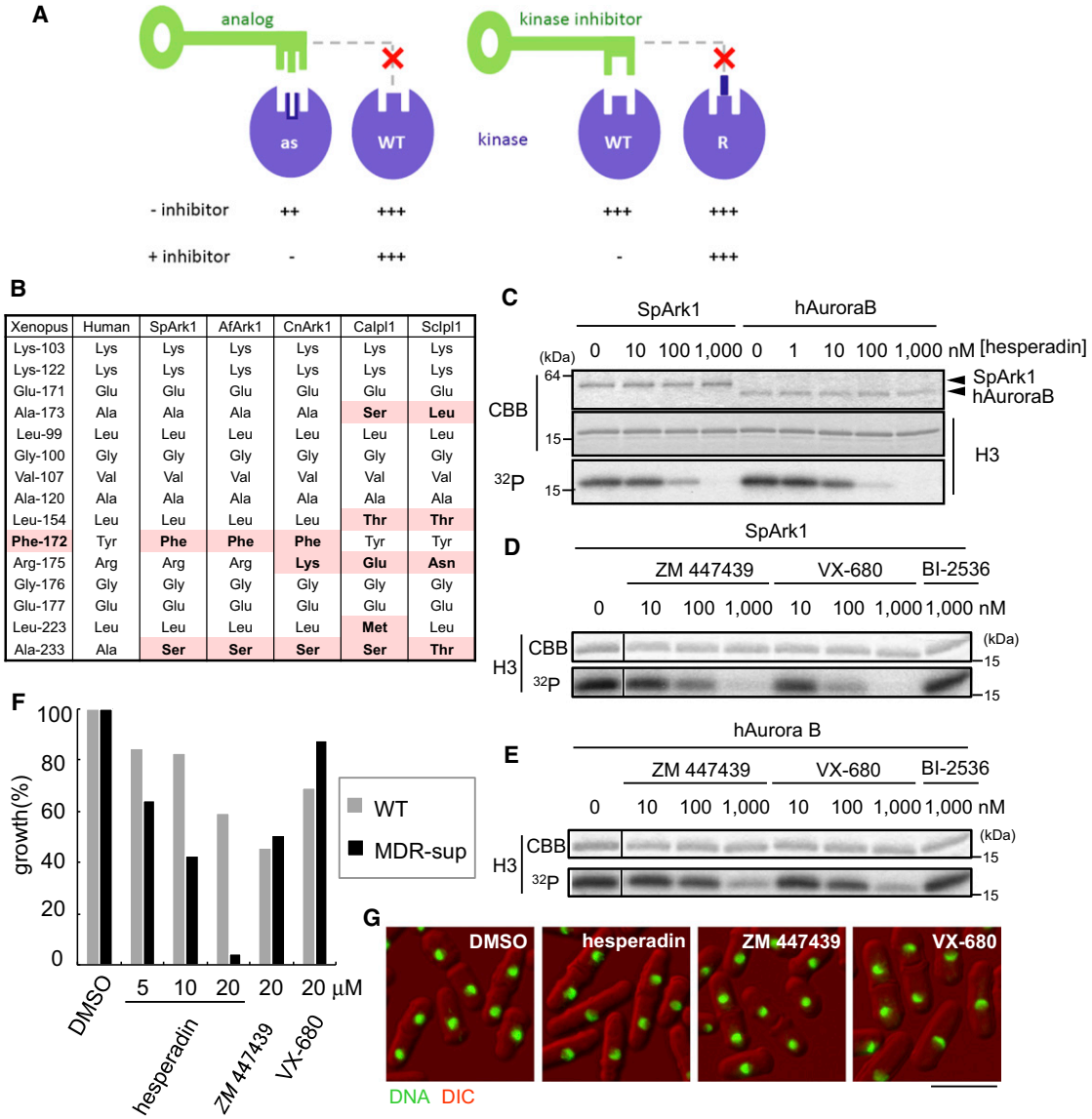
Protein kinases may function more like variable rheostats rather than two-state switches. However, we lack approaches to properly analyze this aspect of kinase-dependent regulation. To address this, we develop a strategy in which a kinase inhibitor is identified using genetics-based screens, kinase mutations that confer resistance are characterized, and dose-dependent responses of isogenic drug-sensitive and resistant cells to inhibitor treatments are compared. This approach has the advantage that function of wild-type kinase, rather than mutants, is examined. To develop this approach, we focus on Ark1, the fission yeast member of the conserved Aurora kinase family. Applying this approach reveals that proper chromosome compaction in fission yeast needs high Ark1 activity, while other processes depend on significantly lower activity levels. Our strategy is general and can be used to examine the functions of other molecular rheostats.

INTRODUCTION

Protein kinases are required for the regulation of a wide range of cellular processes. An emerging theme, from the studies of different kinases, is that these enzymes are not always acting simply as "on" or "off" switches controlling a cellular process. Rather they function as rheostats, with different activity levels controlling specific and distinct phenotypic outcomes. For example, analyses of Plo1, a Polo-like kinase in fission yeast, suggest that processes required for cytokinesis (e.g., septum formation) need higher Plo1 activity than other processes required for cell division (e.g., spindle formation) (Ohkura et al., 1995). In addition, the checkpoint kinase Rad3 is needed for cell cycle arrest and recovery from DNA damage, and analysis of mutants of Rad26, a regulatory subunit of Rad3, suggests that different Rad3 activity levels control arrest and recovery (Wolkow and Enoch, 2002). Further, studies of mutants of Mps1, a conserved kinase that regulates cell division, indicate that key steps in spindle pole body duplication are likely to depend on different kinase activity levels (Schutz and Winey, 1998). However, based on these studies, we are unable to properly establish that kinase activity levels alone control these different phenotypes, as indirect effects, such as changes in kinase localization, cannot be excluded.

Chemical inhibitor-based approaches can be used to control kinase activity and analyze cellular function. However, as kinase inhibitors can have limited or poorly characterized specificity, their use as probes of mechanism can be restricted. A powerful and widely used strategy to address this limitation is to use the "bump-hole" approach, which involves using inhibitors designed to specifically target mutant forms of the kinase of interest but not the wild-type allele or any other kinase (Figure 1A; Bishop et al., 2000). Recently, this approach has been used to examine how different activity levels of cyclin-dependent kinase (CDK) can define independent phases of the cell cycle (Coudreuse and Nurse, 2010) and also to assess the activity thresholds of human Plk1 kinase (Lera and Burkard, 2012). However, drug-sensitive alleles of kinases can have reduced activity when compared to the wild-type enzyme (Bishop et al., 2000; Burkard et al., 2007; Koch and Hauf, 2010), and the mutations in the kinase may not be entirely silent. For example, cells expressing an engineered inhibitor-sensitive *cdc2*-allele (*cdc2-as*) have reduced growth rates, temperature sensitivity, and defects in cell cycle transitions, such as progression through meiosis (Dischinger et al., 2008). In these cases, cells may compensate for reductions in kinase activity levels and complex regulatory networks may adapt, thereby complicating interpretations.

Another alternate strategy, which allows analysis of wild-type kinase function, is to examine dose-dependent phenotypes of a kinase inhibitor in parallel experiments comparing isogenic strains that are either inhibitor-sensitive or carry a mutation in the inhibitor's target that confers resistance but is otherwise silent (Figure 1A). The off-target effects of the inhibitor would be observed in both strains, while the on-target effects would be observed in the drug-sensitive strain alone. To apply this approach, we need to work with model organisms that allow rapid selection and characterization of inhibitor resistance. We also need chemical inhibitors that are active in the model organism. As many diverse chemical scaffolds have been described that inhibit kinases and structures of many kinase-inhibitor complexes are available (Akritopoulou-Zanze and Hajduk, 2009), small focused collections of compounds may



Xenopus	Human	SpArk1	AfArk1	CnArk1	CaIpl1	ScIpl1
Lys-103	Lys	Lys	Lys	Lys	Lys	Lys
Lys-122	Lys	Lys	Lys	Lys	Lys	Lys
Glu-171	Glu	Glu	Glu	Glu	Glu	Glu
Ala-173	Ala	Ala	Ala	Ala	**Ser**	**Leu**
Leu-99	Leu	Leu	Leu	Leu	Leu	Leu
Gly-100	Gly	Gly	Gly	Gly	Gly	Gly
Val-107	Val	Val	Val	Val	Val	Val
Ala-120	Ala	Ala	Ala	Ala	Ala	Ala
Leu-154	Leu	Leu	Leu	Leu	**Thr**	**Thr**
Phe-172	Tyr	**Phe**	**Phe**	**Phe**	Tyr	Tyr
Arg-175	Arg	Arg	Arg	**Lys**	**Glu**	**Asn**
Gly-176	Gly	Gly	Gly	Gly	Gly	Gly
Glu-177	Glu	Glu	Glu	Glu	Glu	Glu
Leu-223	Leu	Leu	Leu	Leu	**Met**	Leu
Ala-233	Ala	**Ser**	**Ser**	**Ser**	**Ser**	**Thr**

Figure 1. Human Aurora Kinase Inhibitors Are Not Active in Fission Yeast

(A) Schematic comparing two approaches that may be used to examine rheostat-like kinase function. (Left) Engineered mutant kinases (as) but not wild-type (WT) kinases are inhibited by inhibitor analogs that contain a sterically bulky substitution. (Right) WT kinases but not inhibitor-resistant kinases (R) are inhibited by specific kinase inhibitors. Predicted kinase activity with or without inhibitors is shown as +++ (high), ++(intermediate), or - (low).

(B) Comparison of predicted hesperadin binding site residues in *Xenopus laevis* (*Xenopus*) Aurora B, human Aurora B (Human), *Schizosaccharomyces pombe* Ark1 (SpArk1), *Aspergillus fumigatus* Ark1 (AfArk1), *Cryptococcus neoformans* Ark1 (CnArk1), *Candida albicans* Ipl1 (CaIpl1), and *Saccharomyces cerevisiae* Ipl1 (ScIpl1). For the *Cryptococcus neoformans* homolog, we identify a protein most similar to SpArk1 using the Basic Local Alignment Search Tool and defined a gene (GeneID: 3257153) as CnArk1.The residues in fungal kinases that are not conserved with human Aurora B are highlighted in pink.

(C–E) Kinase assay with recombinant fission yeast Ark1 (SpArk1) and human Aurora B (hAuroraB). The incorporation of the radioactive phosphate group was visualized by autoradiography (^{32}P), and protein loading was analyzed by staining with Coomassie Brilliant Blue (CBB). A representative data set is shown. The grouping of images from different parts of the same gel is indicated by dividing lines.

(F) Exponentially growing culture (OD = 0.5) of WT (gray) and MDR-sup (black) cells were diluted 50 times in YE4S medium, treated with indicated compounds at the indicated concentrations (μM), and incubated for 14 hr at 32°C. Growth (%) is presented relative to DMSO-treated cells.

(G) Representative images of MDR-sup cells treated with 20 μM of the indicated compounds or DMSO are shown. Scale bars, 10 μm.

See also Figure S1.

be sufficient to identify new probes. Screening strategies, such as those that mimic synthetic lethal screens, can further increase the efficiency in identifying useful chemical tools to examine how dynamic cellular processes are regulated.

Here, we develop and apply this approach to examine rheostat-like kinase-dependent regulation using the fission yeast (*Schizosaccharomyces pombe*), a useful genetically tractable model organism, and focus on Aurora kinase, a conserved

regulator of cell division in eukaryotes. A small collection of kinase inhibitor-like compounds and a synthetic lethal screen was used to identify Arkin-1. We identified a single point mutation in the fission yeast Aurora kinase (Ark1) that confers resistance to Arkin-1 and use dose-dependent inhibition of inhibitor-sensitive and resistant strains to analyze how different levels of Aurora kinase activity control distinct processes required for successful cell division.

RESULTS

Identifying a Chemical Probe for Fission Yeast Aurora Kinase

To determine if available inhibitors of vertebrate Aurora kinases can be used to probe the function of Ark1, the fission yeast Aurora kinase (Petersen et al., 2001), we first examined the X-ray structure of an inhibitor-kinase complex (Sessa et al., 2005). We found that essentially all the residues in *Xenopus* Aurora kinase that interact with the inhibitor, hesperadin, are either identical or very similar to residues in fission yeast and other fungi (Figure 1B). Only one amino acid in the inhibitor-binding pocket is significantly altered (serine-229 in fission yeast replaces alanine-233 in human Aurora kinase). Therefore, we anticipated that available inhibitors of the human Aurora kinases, e.g., hesperadin, ZM 447439, and VX-680, might inhibit the fission yeast kinase.

To examine this, we expressed full-length fission yeast Ark1 and human Aurora B in bacteria and purified active kinase. As anticipated, these compounds inhibited Ark1 in vitro, with potencies that did not appear to be significantly reduced compared to the inhibition of the human kinase (Figures 1C–1E). BI-2536, a human Polo-like kinase 1 (PLK1) inhibitor (Lénárt et al., 2007; Steegmaier et al., 2007), was used as a control and did not inhibit Ark1 (Figures 1D and 1E). However, even though Ark1 is essential in vivo, cell growth of wild-type fission yeast was only partly inhibited by these several selected chemical inhibitors, even at relatively high concentrations (Figure 1F). As reduction in inhibitor efficacy can be due to multidrug resistance (MDR) mechanisms in fission yeast, we used an MDR-sup fission yeast strain that lacks four drug-efflux pumps and one transcription factor. This strain is sensitive to a wide range of chemical inhibitors (Kawashima et al., 2012). While hesperadin (20 μM) inhibited growth of the MDR-sup cells, other inhibitors did not (Figure 1F). As the concentration of hesperadin needed was significantly higher than that needed to inhibit human cells, we needed to validate that toxicity was due to Ark1 inhibition. Genetic knockdown of the Aurora kinase complex in fission yeast show "*cut* (*cell untimely torn*)" phenotypes (Petersen and Hagan, 2003). However, hesperadin treatment did not show any *cut* phenotypes (Figure 1G), suggesting that the major target responsible for the toxicity of hesperadin in fission yeast is not likely to be Aurora kinase. Together, these data indicate that the available inhibitors of vertebrate Aurora kinases cannot be used as probes in fission yeast.

Pathway-Targeted Chemical Screens Identified Arkin-1

As the available inhibitors were active against fission yeast Ark1 in vitro, it is possible that the lack of activity is due to partial cell or nuclear permeability, and more permeable analogs of these inhibitors could be found that are active in cells. However, it is difficult to predict which available inhibitor's analogs should be generated and tested to identify a useful probe. Therefore, to discover inhibitors active in cells, we designed a screen that mimics conventional synthetic lethal genetic screens but, instead of using mutations, selects compounds that are more toxic to a mutant strain relative to a wild-type strain. We anticipated that "hits" from these screens would potentially target proteins involved in the cellular pathway that carried the mutation and are therefore likely to be more specific than inhibitors identified using phenotype-based screens. Such chemical genetic screens have thus far been carried out using budding yeast (Nehil et al., 2007) but, as far as we are aware, not in fission yeast. We focused on condensin, a multisubunit complex needed for error-free cell division, whose function depends on Aurora kinase (Nakazawa et al., 2011; Tada et al., 2011). We introduced a mutation proximal to the Walker A motif in the *cut3* gene (*cut3-KA)*, a subunit of the condensin complex, and found that it results in "partial loss of function" (Figure S2 available online). We introduced this *cut3* allele into the MDR-sup strain and screened an in-house collection of 428 diverse chemicals based on "privileged" chemical scaffolds that are likely to target kinases and ATPases (Peters et al., 2006; Islam et al., 2010). Analysis of MDR-sup and MDR-sup *cut3-KA* strains treated with these compounds (6.7 μM) identified 102 compounds that reduced growth of MDR-sup *cut3-KA* cells by >70%. Of these compounds, only one compound N-(4-morpholinophenyl)-7-(pyridin-2-yl)-7H-pyrrolo[2,3-d]pyrimidin-2-amine showed >15% difference in the growth of MDR-sup and MDR-sup *cut3-KA* cells (Figures 2A and 2B).

We next examined the effect of this hit on another stronger loss of function condensin mutant (*cut3-477)* (Sutani et al., 1999) and found that it was more sensitive than the *cut3-KA* strain (Figure 2C). Importantly, this hit did not have enhanced toxicity for a strain with a mutation in separase (*cut1-22*) (Funabiki et al., 1996), another essential mitotic enzyme (Figure 2C), indicating that this compound selectively targets the chromosome condensation pathway. Unlike hesperadin and the other available Aurora kinase inhibitors, cells treated with this pyrrolopyrimidine revealed a *cut* phenotype, similar to that observed for condensin or Aurora mutants (Figures 3B and 3C; Hirano et al., 1986; Petersen and Hagan, 2003). Together, these data suggest that this compound targets a protein required for proper chromosome condensation.

This pyrrolopyrimidine was structurally similar to INH-32, an inhibitor developed for human Aurora kinase (Figure 2B) that also potently inhibits the Flt3 kinase (Moriarty et al., 2006). Therefore, we examined if our compound targeted Ark1 in vitro and in vivo. We observed dose-dependent inhibition of the phosphorylation of histone H3 by recombinant Ark1 as well as human Aurora B protein (Figure 2D). Next, we examined Ark1 inhibition in cells. As Ark1 activity is mainly restricted to mitosis, we accumulated mitotic cells by inhibiting the proteasome using Velcade and examined the level of histone H3 phosphorylation at Serine 10. We observed a dose-dependent suppression of this phosphorylation (Figures 2E and 3D). In contrast, other human Aurora kinase inhibitors, such as hesperadin, ZM 447439, and VX-680, were not or only weakly active in this assay (Figure 2E). Together, these data indicate that Ark1 is

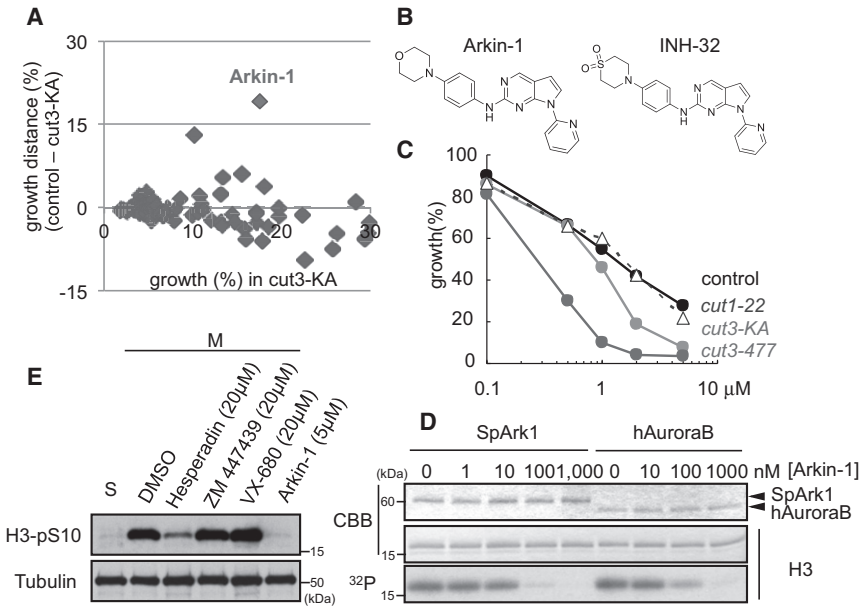


Figure 2. Chemical Synthetic Lethal Screen Using the MDR-sup Fission Yeast Identifies an Ark1 Inhibitor, Arkin-1

(A) Analysis of growth of cells treated with compounds from a 428-member chemical library (6.7 μM). Scatter plot shows the growth of the MDR-sup *cut3-KA* strain (*x* axis) and the difference in growth between MDR-sup (control) and MDR-sup *cut3-KA* strain (y axis). Growth was normalized to DMSO-treated cells. Arkin-1 is indicated.

(B) Chemical structures of Arkin-1 and INH-32 are shown.

(C) Exponentially growing culture (OD = 0.5) of MDR-sup (control: black circle), MDR-sup *cut3-KA* (cut3-KA: light gray circle), MDR-sup *cut1-22* (cut1-22: triangle), and MDR-sup *cut3-477* (cut3-477: dark gray circle) cells were diluted 50 times in YE4S medium and treated with Arkin-1 and incubated for 17 hr at 29°C. Growth (%) is presented relative to DMSO-treated cells.

(D) Kinase assay with recombinant fission yeast Ark1 and human Aurora kinase. The incorporation of the radioactive phosphate group was visualized by autoradiography (^{32}P), and protein loading was analyzed by staining with CBB. A representative data set is shown.

(E) MDR-sup cells were blocked at S-phase by HU to prepare the S-Phase extract (S). To prepare the M-phase extract (M), cells were released from S-phase, incubated for 30 min, and treated with Velcade (40 μM) and indicated Aurora inhibitors for 60 min. The level of histone H3-pS10 was estimated by immunoblot. Tubulin is the loading control.

See also Figure S2.

one of the targets of this compound that we named Arkin-1 (for Ark1 inhibitor-1).

Isolating Arkin-1-Resistant Mutants

To determine if Ark1 is the major physiological target of Arkin-1 in fission yeast, we isolated mutants that are resistant to Arkin-1 using an MDR-sup *cut3-KA* strain. We chemically mutagenized cells and isolated nine resistant clones, which could grow on plates containing Arkin-1 (7.5 μM). As these clones are sensitive to brefeldin A (Figures 3A and S2C) and cycloheximide (Figure S2C), we concluded that these mutations are unlikely to be in MDR genes. We sequenced the *ark1* gene in each drug-resistant clone and found that all nine clones had the same residue mutated, Gly172 (six clones: G172D; two clones: G172S; and one clone: G172C). To confirm that this point mutation in Ark1 is sufficient to confer drug resistance, we constructed a strain in which endogenous *ark1*$^{+}$ gene was replaced by the *ark1-G172D* mutant. Similar to the isolated resistant mutants, the *ark1-GD* mutant was almost completely resistant to Arkin-1 over a range of Arkin-1 concentrations (Figure S3A). Ark1-GD was likely functional, as growth of *ark1-GD* mutant strains was comparable to that of wild-type cells and the localization of Ark1-GD was similar to that of Ark1 (Figures S3A and S3B). We found that abnormal nuclear division as well as the reduction of histone H3 phosphorylation at Serine 10 in Arkin-1-treated cells was almost completely suppressed by the *ark1-GD* mutation (Figures 3B–3D). It is noteworthy that corresponding mutations at this glycine residue in human Aurora kinase have been found to confer resistance to other Aurora inhibitors (hesperadin, ZM 447439, and VX-680) (Balzano et al., 2011; Girdler et al., 2008; Scutt et al., 2009), and it is likely that the Arkin-1, like these other Aurora inhibitors, targets the nucleotide-binding pocket. Together these data indicate that the primary physiological target of Arkin-1 in fission yeast is Ark1, and Arkin-1 is a specific chemical inhibitor of fungal Aurora kinase.

Examining the Roles of Different Ark1 Kinase Activity Levels in Mitosis

Aurora kinase activity gradually increases from prophase to metaphase and then reduces after chromosomes start segregating (Tan and Kapoor, 2011). Nevertheless, Aurora kinase is essential for processes that are needed from the start of mitosis (e.g., regulation of kinetochore-microtubule attachments) and those that may be completed later in mitosis (e.g., chromosome condensation). Therefore, Aurora-dependent regulation of these processes may depend on different levels of kinase activity. Recently, a study using several INCENP mutants in chicken DT40 cells has suggested that higher levels of Aurora B kinase activity are required for completing cytokinesis (Xu et al., 2010). However, as *ark1* mutants show *cut* phenotypes, fission yeast Ark1 is not required for completing cytokinesis. Moreover, requirement of different Aurora kinase activity levels for kinetochore-microtubule attachments or chromosome condensation has not been addressed. To examine Ark1 kinase activity-dependent regulation of chromosome segregation, we used Arkin-1 and parallel analyses of dose-dependent phenotypes in inhibitor-sensitive and resistance cells. As Arkin-1 did not work in the wild-type background, we used the MDR-sup strain for all the experiments (Figure S2D).

We first analyzed the level of phosphorylation of a key substrate (Histone H3) over a range of Arkin-1 concentrations. Substrate phosphorylation was reduced by ~40% at 2 μM

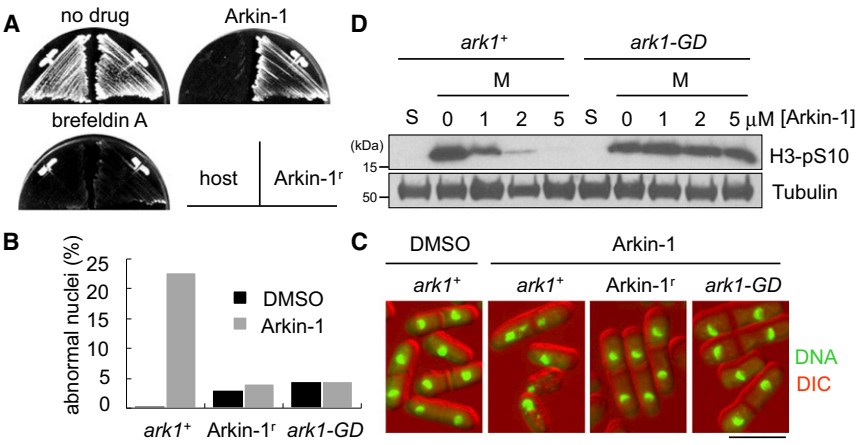


Figure 3. Identification of an Arkin-1-Resistant Mutant

(A) MDR-sup *cut3-KA* cells (host) and Arkin-1-resistant cells (Arkin-1^r) were streaked on YE4S plate or YE4S plate containing Arkin-1 (7.5 μM) or brefeldin A (2 μM) and incubated at 29°C.

(B and C) The frequency of abnormal chromosome segregation was examined in MDR-sup *cut3-KA* cells (*ark1*$^+$), Arkin-1-resistant clone (MDR-sup *cut3-KA* background) (Arkin-1^r), and MDR-sup *cut3-KA ark1-GD* (*ark1-GD*) cells after 5 μM Arkin-1 (or DMSO) treatment in asynchronous culture (2.5 hr at 32°C [n > 250]). Representative images are shown. Scale bars, 10 μm.

(D) MDR-sup *cut3-KA* cells, with or without *ark1-GD* mutation, were blocked at S-phase by HU to prepare the S-Phase extract (S). To prepare the M-phase extract (M), these strains were released from S-phase, incubated for 30 min, and treated with 10 μM Velcade and Arkin-1 for 60 min. The level of histone H3-pS10 was estimated by immunoblot. Tubulin is the loading control.

See also Figure S3.

Arkin-1 and ~90% at 5 μM or 7.5 μM Arkin-1 (Figure S4). We also analyzed the localization of Ark1 in Arkin-1-treated cells and found that the centromeric localization of Ark1 was intact in 7.5 μM Arkin-1-treated cells (Figure S3C). We next examined Ark1's spindle assembly checkpoint (SAC) function by using nocodazole to induce microtubule depolymerization and activate the SAC and then treating with Arkin-1. In addition, we analyzed kinetochore localization of Mad2 at kinetochores, an indicator of SAC activation. To examine Ark1's function in correcting errors in kinetochore-microtubule attachments, we analyzed the frequency of improper attachments, including syntelic and merotelic attachments, by tracking chromosome II segregation using *cen2*-green fluorescent protein (GFP) (Hauf et al., 2007). To examine Ark1's function in chromosome condensation, we measured the frequency of cells that have reduced chromosome compaction compared to controls but have normally separated *cen2*-GFP signals during anaphase. Our measure of chromosome condensation defects is likely to be an underestimate, as we do not include cells with improper chromosome attachments.

Treatment with high doses of Arkin-1 (7.5 μM) resulted in SAC defects with loss of Mad2 localization, defects in correcting errors in kinetochore-microtubule attachments, and improper chromosome condensation (Figures 4A–4D). All these defects were suppressed in *ark1-GD* strain (Figures 4A–4D), suggesting that observed phenotypes depend on Ark1's kinase activity. Low doses of Arkin-1 (2 μM) treatment resulted in obvious defects of chromosome condensation (46% ± 4%, two independent experiments). Interestingly, errors in kinetochore-microtubule attachments were very low (3.1%), and SAC arrest/Mad2 localization was unaltered under these conditions (Figures 4A–4D). An intermediate dose of Arkin-1 (5 μM) treatment shows partial defects in SAC arrest/Mad2 localization and error correction and severe defects in chromosome condensation (Figure 4A–4D). Together, these data suggest that proper chromosome condensation requires higher levels of Ark1 kinase activity than that needed for the spindle assembly checkpoint or to establish proper kinetochore-microtubule attachments.

To further characterize defects of chromosome compaction induced by low doses of Arkin-1, we quantitatively analyzed the separation of centromeres (indicated by *imr3*-tdTomato; Sakuno et al., 2009) or chromosome arm regions (indicated by *arm*-GFP, centromeric side of the ribosomal DNA [rDNA] locus; Tada et al., 2011) of chromosome III (ch3) during anaphase. In addition, as Aurora kinase is required for efficient separation of nucleoli or rDNA in budding yeast (Sullivan et al., 2004), we also analyzed the separation of the nucleolus, indicated by cyan fluorescent protein (CFP)-fused Gar1 (Tada et al., 2011). Consistent with the results that attachment defects of centromere II were only rarely observed at low doses of Arkin-1 (Figures 4C and 4D), separation of ch3-centromeres in Arkin-1-treated cells was comparable to DMSO-treated controls (Figure 5A). In contrast, separation of ch3-arm and nucleolus was significantly less efficient at low doses of Arkin-1 (Figures 5B and 5D). These defects were suppressed in *ark1-GD* cells, even at the highest concentration (7.5 μM) of Arkin-1, confirming specificity (Figures S5A and S5B).

Ark1 phosphorylates the amino-terminal fragment of Cnd2 (homolog of human Barren), and the phosphomimetic mutant *cnd2-3E* suppresses the growth defect by Ark1 inhibition (Nakazawa et al., 2011; Tada et al., 2011). Therefore, we examined if reduction of Cnd2 phosphorylation is the major reason underlying the observed chromosome compaction defects. If true, low dose of Arkin-1 treatment should not enhance chromosome compaction defects in the *cnd2-3E* mutant background. However, Arkin-1 treatment significantly reduced separation of chromosome arms and nucleoli (Figures 5E, 5F, S5E, and S5F), suggesting that Ark1-dependent phosphorylation of factors other than Cnd2 is also important for proper chromosome compaction.

Mitotic phosphorylation of substrates depends on a balance between kinase and phosphatase activity (Bollen et al., 2009). Protein phosphatase 1 (PP1) opposes Aurora-dependent regulation of the SAC (in fission yeast; Meadows et al., 2011; Vanoosthuyse and Hardwick, 2009) and chromosome-microtubule

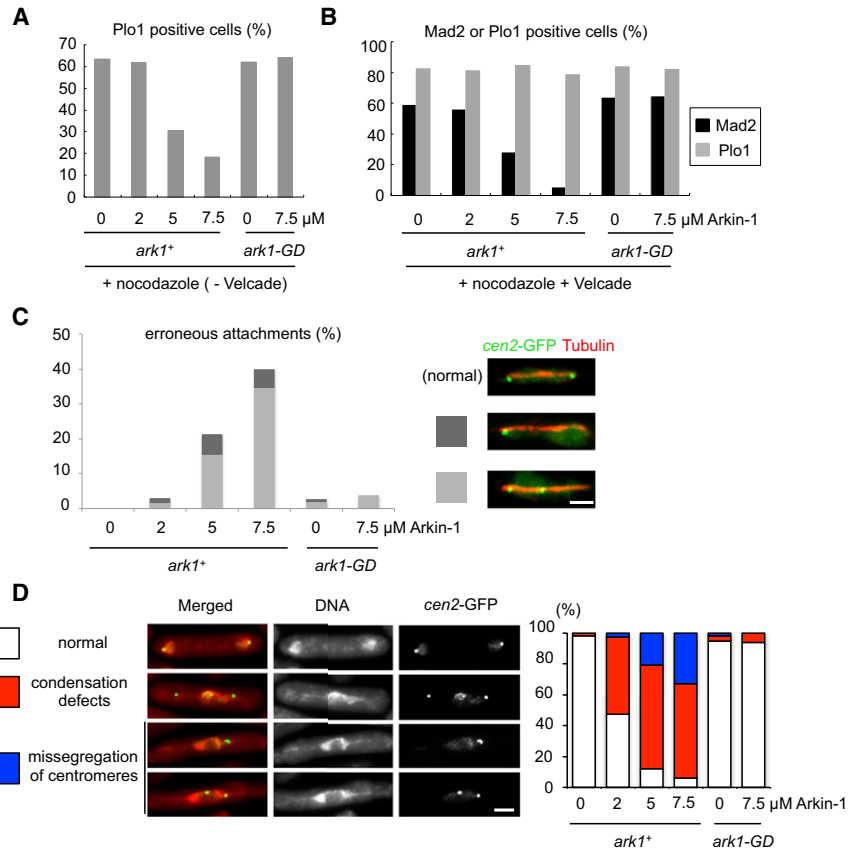


Figure 4. Examining the Contributions of the Kinase Activity of Ark1 during Mitosis

Duration of Arkin-1 treatment is 1 hr for all the experiments.

(A and B) Synchronized MDR-sup *plo1-mYFP mad2-mcherry* cells, with or without *ark1-GD* mutation, was treated with nocodazole (A) or nocodazole/Velcade (B) for 45 min, and then the indicated concentrations of Arkin-1 were added. Percentage of cells with strong dot-like signals of Plo1-mYFP (gray) or Mad2-mcherry (black) were analyzed ($n > 200$). The Plo1 signals at spindle pole bodies, which depend on CDK activity, are an indicator of (pro)metaphase cells.

(C) Synchronized MDR-sup *mcherry-atb2 cen2-GFP* cells, with or without *ark1-GD* mutation, were treated with the indicated concentrations of Arkin-1. The percentage of erroneous kinetochore-microtubule attachments in anaphase cells were analyzed ($n > 100$). Representative images are shown. Scale bars, 2 μm.

(D) Synchronized MDR-sup *plo1-mYFP cen2-GFP* cells were treated with the indicated concentrations of Arkin-1. The percentage of each phenotype (white: normal; red: condensation defects; blue: missegregation of centromeres) in Plo1-negative anaphase cells were analyzed ($n > 150$). Representative images are shown. Scale bars, 2 μm.

See also Figure S4.

attachment (in human cells; Liu et al., 2010). Consistent with these reports, deletion of Dis2, the kinetochore-localized isoform of PP1 (Alvarez-Tabarés et al., 2007), suppressed delocalization of Mad2 from kinetochore and error correction defects in Arkin-1-treated MDR-sup fission yeast cells (Figures S5C and S5D). Because the phosphatases counteracting Aurora-dependent chromosome compaction are unknown, we examined if Dis2 is involved in this regulation. Strikingly, deletion of Dis2 suppressed the defects of ch3-arm and nucleolus separation in cells treated with low doses of Arkin-1 (Figures 5C and 5D). These data suggest that PP1 counteracts Aurora-dependent chromosome compaction in fission yeast.

DISCUSSION

In this study, we present a strategy to investigate how distinct cellular phenotypes can be regulated via differences in protein kinase activity levels. Our strategy involves the identification of a cell-permeable kinase inhibitor, selection and characterization of mutations in the inhibitor's target, and analysis of dose-dependent phenotypes in inhibitor-sensitive or resistant isogenic cells (Figure 1A). This strategy has advantages over other chemical genetic strategies, as concerns relating to inhibitor specificity or compensation/adaptation in cells with partially impaired kinase can be addressed. In the case of Ark1, the bump-hole approach has begun to be developed and "analog sensitive" (ark1-as2 or ark1-as3) alleles have been described (Koch and Hauf, 2010). The strains expressing these ark1 alleles reveal some chromosome segregation errors, even in the absence of inhibitors (Koch and Hauf, 2010), and generating completely silent alleles appears to be challenging. Therefore, the effective use of this powerful strategy remains restricted. Our approach examining the wild-type Ark1 provides a useful alternate and, at this stage, is likely to allow for stronger interpretations of experiments analyzing the rheostat-like functions of Ark1 during cell division.

While potentially general, our strategy depends on identifying resistance-conferring mutations in the inhibitor's target that do not disrupt function. While it is extremely difficult to establish that the mutant has no phenotype at all (Yun et al., 2008), it is easier to determine if there is a phenotype in the cellular process we wish to examine. Moreover, as these mutations interfere with drug binding rather than ATP-binding, a function essential for kinase activity, it is more likely that mutations can be identified that confer drug resistance but preserve overall function. Consistent with this assumption, we identified Arkin-1 resistance, conferring mutations that did not significantly reduce growth and had a less than 5% defect in chromosome segregation. Examining other mutations, such as *ark1* (*G172L*) that, based on studies with other inhibitors, (Scutt et al., 2009) should also confer Arkin-1 resistance, may yield strains that have even milder phenotypes.

The successful application of our approach also depends on finding chemical inhibitors that are active in cells. One strategy to identify inhibitors for a kinase of interest is to use structural data and analyze the conservation of residues in the

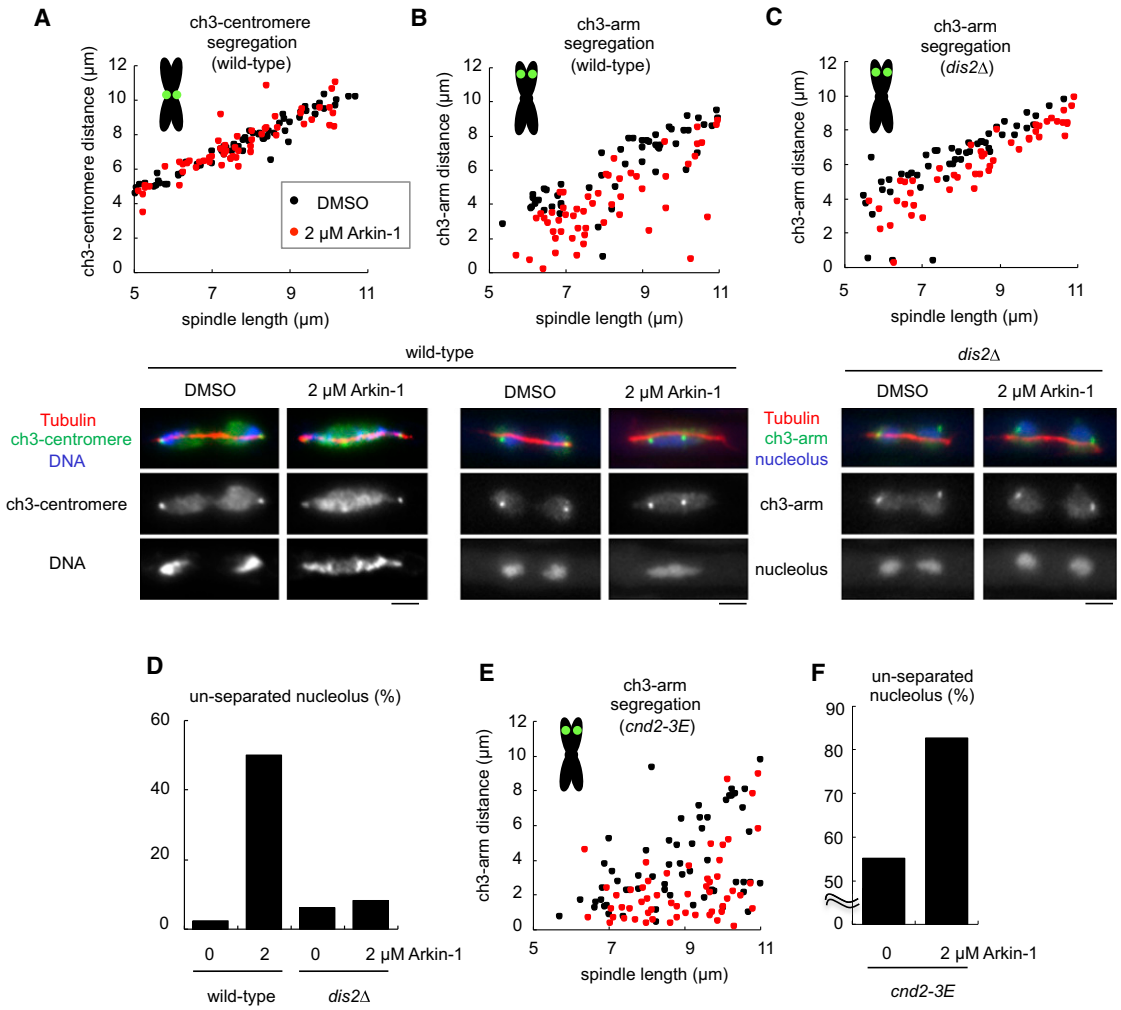


Figure 5. Proper Separation of Chromosome Arms and Nucleoli Needs High Levels of Ark1 Activity

Synchronized MDR-sup cells (A and B: wild-type; C and D: *dis2Δ*; and E and F: *cnd2-3E*) were treated with 2 μM Arkin-1 (or DMSO) for 1 hr at 32°C. Tubulin, *imr3*-tdTomato (ch3-centromere) or *arm*-GFP (ch3-arm), Gar1-CFP (nucleoli), and DNA were imaged.

(A–C and E) The distance of *imr3*-tdTomato (ch3-centromere) or *arm*-GFP (ch3-arm) dots and spindle length in anaphase cells were analyzed ($n > 48$). In the graphs, each circle indicates one anaphase cell treated with 2 μM Arkin-1 (red) or DMSO (black). Representative images are shown. Scale bars, 2 μm.

(D and F) Frequency of unseparated nucleolus indicated by Gar1-CFP in DMSO or 2 μM Arkin-1-treated cells was analyzed. As nucleoli were not yet separated in ~50% early anaphase DMSO-treated cells (spindle length < 7 μm), late anaphase cells (7 μm < spindle length < 11 μm) were analyzed ($n > 33$).

See also Figure S5.

drug-binding pockets to determine if an available probe may be active. However, we find that available Aurora kinase inhibitors are inactive in fission yeast cells, despite the drug-binding pockets of the fission yeast and human Aurora kinases being very similar. For other highly conserved kinases, such as Polo-like kinase, analysis of drug-binding sites of BI2536, a specific inhibitor of human PLK1, reveals even greater divergence between the fungal and human kinase (Figure S1A). Consistent with these analyses, we found that fission yeast strains were not sensitive to extremely high concentrations of BI-2536 (>10 μM) compared to active dose for human cells (~10 nM) (Figure S1B). Together, these data suggest that available inhibitors that target vertebrate kinases may not work in fission yeast, most likely due to differences in cell permeability or divergence in the drug-binding pockets. To address this limitation, the deletion of additional genes to increase cell permeability may be considered but is not likely to be very practical, due to reduced strain viability and restrictions in additional genetic manipulations of the MDR-sup strain we have recently developed. We believe that, while the available inhibitors may not work, their analogs may well be effective and can be efficiently identified using "chemical synthetic lethal" screens, as used here. This screening strategy has the advantage that it is likely to identify compounds that are selective for the target pathway, addressing at an early stage of the discovery process a key limitation of chemical inhibitors. Furthermore, we can increase the fidelity of this selection by testing drug sensitivity of another mutant in the target pathway (e.g., *cut3-477*) and by the lack of increased activity against an unrelated mutant (e.g., *cut1-22*) (Figure 2C).

Dose-dependent inhibition of Ark1 in isogenic drug-sensitive and resistant strains revealed that proper chromosome compaction, which is essential for efficient separation of chromosome arms and nucleoli during anaphase, requires higher levels of Ark1 kinase activity than that needed either for checkpoint signaling or for establishing proper kinetochore-microtubule attachments. The SAC and the error correction mechanisms must be active early during mitosis (prophase-prometaphase), while proper chromosome compaction could be achieved by late mitosis and needs to be completed prior to anaphase (metaphase-anaphase). Therefore, the dependence of these processes on different levels of kinase activity suggests that the changes in Aurora kinase activity during mitosis can help to establish temporal order during cell division.

Our observations can be explained by a model in which phosphorylation of substrates located proximal to the site where the kinase is localized is more efficient than the modification of substrates that are more distal (Fuller et al., 2008; Wang et al., 2011). Ark1 substrates regulating Mad2 recruitment or kinetochore-microtubule attachments are likely to be enriched at the kinetochore, which is proximal to the centromere, where most Ark1 accumulates prior to anaphase. In contrast, Ark1 substrates regulating chromosome compaction (e.g., condensins) are likely to localize all along chromosome arms, which can extend microns away from the centromere and thus are displaced from the majority of Ark1. Therefore, regulation of kinetochore function may require lower levels of kinase activity than that needed for substrates distributed along the entire chromosome. Alternative models, such as differences between chromosome and kinetochore substrate affinities or even local substrate concentrations, may also explain our observations. Additional studies will have to be designed to further examine these possibilities. How chromosomes are properly compacted for cell division remains poorly understood. Our data indicate that phosphoregulation of the condensin subunit Cnd2 alone does not account for the observed phenotype, and at least one more unknown factor must be regulated by Ark1 and PP1 to achieve proper chromosome compaction. Interestingly, condensin-independent chromosome compaction regulated by PP1 has been suggested by a study examining cell division in chicken DT40 cells (Vagnarelli et al., 2006), and this may be relevant to the unknown factor. Finding this factor, which is likely to be conserved among eukaryotes, is an important goal, and fission yeast may be well-suited for these studies.

The level of substrate phosphorylation depends on a balance in kinase and phosphatase activity. In humans and in fission yeast cells, PP1 targets to multiple sites, including nucleolus, kinetochores, and chromatin (Alvarez-Tabarés et al., 2007; Trinkle-Mulcahy et al., 2006), suggesting that PP1 dynamically regulates multiple cellular events. Our data indicate that PP1 counteracts Aurora-dependent regulation of chromosome condensation. Understanding the precise contribution of phosphatases to rheostat-like regulation is difficult, due to the fact that diversity in phosphatase function is achieved by distinct regulatory subunits in different holoenzyme complexes that share a common catalytic subunit. Developing good compounds that selectively inhibit a particular phosphatase holoenzyme complex has been challenging, as protein-protein interactions need to be disrupted, something that is not readily achieved by low molecular weight cell permeable small molecules (Arkin and Wells, 2004). Chemical synthetic screens for phosphatase inhibitors may be successful, particularly if the compound libraries tested are large, diverse, and include scaffolds with features that can increase the likelihood that protein-protein interactions are inhibited (Morelli et al., 2011). These chemical probes, along with characterized resistance-conferring mutant forms of the phosphatase, will be useful for examining how kinases and phosphatases quantitatively communicate and regulate cell division dynamics.

Our approach could be applied to examine the rheostat-like regulation of dynamic and essential cellular processes by other kinases, such as Rad3 or Mps1. In principle, our strategy can also be applied to other regulatory enzymes, such as histone-modifying enzymes, for which several different chemical inhibitors have recently been reported (Cole, 2008), and therefore, identifying probes active in fission yeast should be feasible. In addition to serving as probes of cellular mechanisms, it is possible that the chemical inhibitors we identify and validate will provide starting points for developing new antifungal drugs.

SIGNIFICANCE

Studies of different kinases suggest that these enzymes are not acting simply as "on" or "off" switches controlling a cellular process. Rather they function as variable rheostats, with different activity levels controlling specific and distinct phenotypic outcomes. We report a proof-of-concept study developing a robust chemical biology strategy to analyze rheostat-like protein kinase-dependent regulation. This approach involves the use of genetic screens to identify a chemical probe for a kinase of interest, characterization of mutations in the kinase that confer resistance, and then comparisons between dose-dependent responses of isogenic drug-sensitive and resistant cells to inhibitor treatment. To develop the approach, we focused on Aurora kinase, a conserved regulator of cell division in eukaryotes. We used a synthetic lethal screen to identify Arkin-1, a specific inhibitor of fission yeast Aurora kinase (Ark1), and characterized a resistance-conferring point mutation in Ark1. Parallel experiments analyzing the responses of drug-sensitive and resistant cells to controlled amounts of Arkin-1 revealed that efficient separation of chromosome arms and nucleoli, which depend on proper chromosome compaction, needs high levels of Ark1 activity. In contrast, regulation of checkpoint signaling and chromosome-microtubule attachments needs significantly less kinase activity. Further chemical genetic experiments using low doses of Arkin-1 suggested that Ark1-dependent phosphoregulation of an unknown factor, other than the condensin subunit Cnd2, and the phosphatase protein phosphatase 1 are needed to achieve proper chromosome compaction. Our approach could be applied to examine the rheostat-like regulation of dynamic and essential cellular processes by other kinases. In addition to serving as probes of cellular mechanisms, it is possible that the chemical inhibitors we identify and validate will provide starting points for developing new antifungal drugs.

EXPERIMENTAL PROCEDURES

Schizosaccharomyces pombe Strains

All strains used are listed in Table S1. Standard growth conditions and methods were used (Moreno et al., 1991). All experiments were performed in yeast extract (YE) medium containing adenine, leucine, uridine, and histidine. The PCR-based gene targeting (Bähler et al., 1998) was used to construct gene disruptants and fluorescent protein–tagged strains with selection marker gene cassettes. The MDR-sup strain is an engineered drug-sensitive fission yeast, in which five genes were deleted (*pap1Δ bfr1Δ pmd1Δ mfs1Δ caf5Δ*) (Kawashima et al., 2012).

Immunoblotting

Total protein was extracted using glass beads (Moreno et al., 1991), separated by SDS-PAGE, and transferred to membrane filters. For primary antibodies, antiphospho-Histone H3 (Ser10) (1:2,000, Millipore) and TAT-1 (1:2,000; a gift from K. Gull; Woods et al., 1989) were used.

Chemical Synthetic Lethal Screen

AI61 and AI82 strains (Table S1) were used for the chemical genetic screen. Logarithmically growing cells (optical density [OD] = 0.5) were diluted ten times, mixed with compounds (6.7 μM) and YE medium (total volume: 50 μl per well), and incubated for 12 hr at 29°C. Multidrop Combi (Thermo Scientific) was used to dispense the cells into wells of the 384-plate (Greiner clear flat bottom PS plate). The growth was measured by microtiter plate reader (Perkin-Elmer EnVision, 590 nm filter). For calculation of growth ratio, OD values of each well were divided by that of control well incubated with DMSO.

Isolation of Arkin-1-Resistant Mutants

AI164 strain (Table S1) was mutagenized by treatment with 1-methyl-3-nitro-1-nitrosoguanidine (NTG) (25 μg/ml, 75% viability compared to no NTG condition) in Tris maleic acid buffer (50 mM Tris, 50 mM maleic acid, 7.5 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4 \cdot 7H_2O$, pH 6.0) for 30 min and incubated in YE medium for >3 hr, spread onto plates containing 7.5 μM Arkin-1. Nine Arkin-1-resistant mutants were isolated from $\sim 10^6$ cells.

Synchronization of MDR-sup Cells

MDR-sup cells were blocked at S-phase using hydroxyurea (HU) and released from S phase by washing out HU at 32°C. Nocodazole (15 μM) or Velcade (40 μM) was added 30 min after release to activate the spindle checkpoint with unattached kinetochores or to prevent metaphase-anaphase transition, respectively.

Microscopy

Cells were fixed using methanol, stained with DAPI, imaged by a microscope (Axioplan 2; Carl Zeiss), and processed with MetaMorph software (Molecular Devices), as described previously (Kawashima et al., 2012).

ACCESSION NUMBERS

The NCBI accession number for the CnArk1 reported in this paper is 3257153.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.01.003.

ACKNOWLEDGMENTS

We thank Fraser Glickman for the use of the Rockefeller University High Throughput Screening Resource Center, Andrea Musacchio and Yoshinori Watanabe for providing strains and plasmids, and Kabirul Islam and Sudir Kashap for inhibitor synthesis. This work was supported by JSPS Postdoctoral Fellowships for Research Abroad (to S.A.K. and A.T.), by M. J. and H. Kravis (to S.A.K.), by The Beast Cancer Research Foundation, the Wellcome Trust, and The Rockefeller University (to P.N.), and by the National Institutes of Health/National Institute of General Medical Sciences (GM098579 to T.M.K.).

Received: September 26, 2012
Revised: December 4, 2012
Accepted: January 2, 2013
Published: February 21, 2013

REFERENCES

Akritopoulou-Zanze, I., and Hajduk, P.J. (2009). Kinase-targeted libraries: the design and synthesis of novel, potent, and selective kinase inhibitors. Drug Discov. Today *14*, 291–297.

Alvarez-Tabarés, I., Grallert, A., Ortiz, J.M., and Hagan, I.M. (2007). Schizosaccharomyces pombe protein phosphatase 1 in mitosis, endocytosis and a partnership with Wsh3/Tea4 to control polarised growth. J. Cell Sci. *120*, 3589–3601.

Arkin, M.R., and Wells, J.A. (2004). Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. Nat. Rev. Drug Discov. *3*, 301–317.

Bähler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., 3rd, Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast *14*, 943–951.

Balzano, D., Santaguida, S., Musacchio, A., and Villa, F. (2011). A general framework for inhibitor resistance in protein kinases. Chem. Biol. *18*, 966–975.

Bishop, A.C., Ubersax, J.A., Petsch, D.T., Matheos, D.P., Gray, N.S., Blethrow, J., Shimizu, E., Tsien, J.Z., Schultz, P.G., Rose, M.D., et al. (2000). A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature *407*, 395–401.

Bollen, M., Gerlich, D.W., and Lesage, B. (2009). Mitotic phosphatases: from entry guards to exit guides. Trends Cell Biol. *19*, 531–541.

Burkard, M.E., Randall, C.L., Larochelle, S., Zhang, C., Shokat, K.M., Fisher, R.P., and Jallepalli, P.V. (2007). Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. Proc. Natl. Acad. Sci. USA *104*, 4383–4388.

Cole, P.A. (2008). Chemical probes for histone-modifying enzymes. Nat. Chem. Biol. *4*, 590–597.

Coudreuse, D., and Nurse, P. (2010). Driving the cell cycle with a minimal CDK control network. Nature *468*, 1074–1079.

Dischinger, S., Krapp, A., Xie, L., Paulson, J.R., and Simanis, V. (2008). Chemical genetic analysis of the regulatory role of Cdc2p in the S. pombe septation initiation network. J. Cell Sci. *121*, 843–853.

Fuller, B.G., Lampson, M.A., Foley, E.A., Rosasco-Nitcher, S., Le, K.V., Tobelmann, P., Brautigan, D.L., Stukenberg, P.T., and Kapoor, T.M. (2008). Midzone activation of aurora B in anaphase produces an intracellular phosphorylation gradient. Nature *453*, 1132–1136.

Funabiki, H., Kumada, K., and Yanagida, M. (1996). Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. EMBO J. *15*, 6617–6628.

Girdler, F., Sessa, F., Patercoli, S., Villa, F., Musacchio, A., and Taylor, S. (2008). Molecular basis of drug resistance in aurora kinases. Chem. Biol. *15*, 552–562.

Hauf, S., Biswas, A., Langegger, M., Kawashima, S.A., Tsukahara, T., and Watanabe, Y. (2007). Aurora controls sister kinetochore mono-orientation and homolog bi-orientation in meiosis-I. EMBO J. *26*, 4475–4486.

Hirano, T., Funahashi, S., Uemura, T., and Yanagida, M. (1986). Isolation and characterization of Schizosaccharomyces pombe cutmutants that block nuclear division but not cytokinesis. EMBO J. *5*, 2973–2979.

Islam, K., Chin, H.F., Olivares, A.O., Saunders, L.P., De La Cruz, E.M., and Kapoor, T.M. (2010). A myosin V inhibitor based on privileged chemical scaffolds. Angew. Chem. Int. Ed. Engl. *49*, 8484–8488.

Kawashima, S.A., Takemoto, A., Nurse, P., and Kapoor, T.M. (2012). Analyzing fission yeast multidrug resistance mechanisms to develop a genetically tractable model system for chemical biology. Chem. Biol. *19*, 893–901.

Koch, A., and Hauf, S. (2010). Strategies for the identification of kinase substrates using analog-sensitive kinases. Eur. J. Cell Biol. *89*, 184–193.

Lénárt, P., Petronczki, M., Steegmaier, M., Di Fiore, B., Lipp, J.J., Hoffmann, M., Rettig, W.J., Kraut, N., and Peters, J.M. (2007). The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. Curr. Biol. *17*, 304–315.

Lera, R.F., and Burkard, M.E. (2012). High mitotic activity of Polo-like Kinase 1 is required for chromosome segregation and genomic integrity in human epithelial cells. J. Biol. Chem. *287*, 42812–42825.

Liu, D., Vleugel, M., Backer, C.B., Hori, T., Fukagawa, T., Cheeseman, I.M., and Lampson, M.A. (2010). Regulated targeting of protein phosphatase 1 to the outer kinetochore by KNL1 opposes Aurora B kinase. J. Cell Biol. *188*, 809–820.

Meadows, J.C., Shepperd, L.A., Vanoosthuyse, V., Lancaster, T.C., Sochaj, A.M., Buttrick, G.J., Hardwick, K.G., and Millar, J.B. (2011). Spindle checkpoint silencing requires association of PP1 to both Spc7 and kinesin-8 motors. Dev. Cell *20*, 739–750.

Morelli, X., Bourgeas, R., and Roche, P. (2011). Chemical and structural lessons from recent successes in protein-protein interaction inhibition (2P2I). Curr. Opin. Chem. Biol. *15*, 475–481.

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. *194*, 795–823.

Moriarty, K.J., Koblish, H.K., Garrabrant, T., Maisuria, J., Khalil, E., Ali, F., Petrounia, I.P., Crysler, C.S., Maroney, A.C., Johnson, D.L., and Galemmo, R.A., Jr. (2006). The synthesis and SAR of 2-amino-pyrrolo[2,3-d]pyrimidines: a new class of Aurora-A kinase inhibitors. Bioorg. Med. Chem. Lett. *16*, 5778–5783.

Nakazawa, N., Mehrotra, R., Ebe, M., and Yanagida, M. (2011). Condensin phosphorylated by the Aurora-B-like kinase Ark1 is continuously required until telophase in a mode distinct from Top2. J. Cell Sci. *124*, 1795–1807.

Nehil, M.T., Tamble, C.M., Combs, D.J., Kellogg, D.R., and Lokey, R.S. (2007). Uncovering genetic relationships using small molecules that selectively target yeast cell cycle mutants. Chem. Biol. Drug Des. *69*, 258–264.

Ohkura, H., Hagan, I.M., and Glover, D.M. (1995). The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. Genes Dev. *9*, 1059–1073.

Peters, U., Cherian, J., Kim, J.H., Kwok, B.H., and Kapoor, T.M. (2006). Probing cell-division phenotype space and Polo-like kinase function using small molecules. Nat. Chem. Biol. *2*, 618–626.

Petersen, J., and Hagan, I.M. (2003). S. pombe aurora kinase/survivin is required for chromosome condensation and the spindle checkpoint attachment response. Curr. Biol. *13*, 590–597.

Petersen, J., Paris, J., Willer, M., Philippe, M., and Hagan, I.M. (2001). The S. pombe aurora-related kinase Ark1 associates with mitotic structures in a stage dependent manner and is required for chromosome segregation. J. Cell Sci. *114*, 4371–4384.

Sakuno, T., Tada, K., and Watanabe, Y. (2009). Kinetochore geometry defined by cohesion within the centromere. Nature *458*, 852–858.

Schutz, A.R., and Winey, M. (1998). New alleles of the yeast MPS1 gene reveal multiple requirements in spindle pole body duplication. Mol. Biol. Cell *9*, 759–774.

Scutt, P.J., Chu, M.L., Sloane, D.A., Cherry, M., Bignell, C.R., Williams, D.H., and Eyers, P.A. (2009). Discovery and exploitation of inhibitor-resistant aurora and polo kinase mutants for the analysis of mitotic networks. J. Biol. Chem. *284*, 15880–15893.

Sessa, F., Mapelli, M., Ciferri, C., Tarricone, C., Areces, L.B., Schneider, T.R., Stukenberg, P.T., and Musacchio, A. (2005). Mechanism of Aurora B activation by INCENP and inhibition by hesperadin. Mol. Cell *18*, 379–391.

Steegmaier, M., Hoffmann, M., Baum, A., Lénárt, P., Petronczki, M., Krssák, M., Gürtler, U., Garin-Chesa, P., Lieb, S., Quant, J., et al. (2007). BI 2536, a potent and selective inhibitor of polo-like kinase 1, inhibits tumor growth in vivo. Curr. Biol. *17*, 316–322.

Sullivan, M., Higuchi, T., Katis, V.L., and Uhlmann, F. (2004). Cdc14 phosphatase induces rDNA condensation and resolves cohesin-independent cohesion during budding yeast anaphase. Cell *117*, 471–482.

Sutani, T., Yuasa, T., Tomonaga, T., Dohmae, N., Takio, K., and Yanagida, M. (1999). Fission yeast condensin complex: essential roles of non-SMC subunits for condensation and Cdc2 phosphorylation of Cut3/SMC4. Genes Dev. *13*, 2271–2283.

Tada, K., Susumu, H., Sakuno, T., and Watanabe, Y. (2011). Condensin association with histone H2A shapes mitotic chromosomes. Nature *474*, 477–483.

Tan, L., and Kapoor, T.M. (2011). Examining the dynamics of chromosomal passenger complex (CPC)-dependent phosphorylation during cell division. Proc. Natl. Acad. Sci. USA *108*, 16675–16680.

Trinkle-Mulcahy, L., Andersen, J., Lam, Y.W., Moorhead, G., Mann, M., and Lamond, A.I. (2006). Repo-Man recruits PP1 gamma to chromatin and is essential for cell viability. J. Cell Biol. *172*, 679–692.

Vagnarelli, P., Hudson, D.F., Ribeiro, S.A., Trinkle-Mulcahy, L., Spence, J.M., Lai, F., Farr, C.J., Lamond, A.I., and Earnshaw, W.C. (2006). Condensin and Repo-Man-PP1 co-operate in the regulation of chromosome architecture during mitosis. Nat. Cell Biol. *8*, 1133–1142.

Vanoosthuyse, V., and Hardwick, K.G. (2009). A novel protein phosphatase 1-dependent spindle checkpoint silencing mechanism. Curr. Biol. *19*, 1176–1181.

Wang, E., Ballister, E.R., and Lampson, M.A. (2011). Aurora B dynamics at centromeres create a diffusion-based phosphorylation gradient. J. Cell Biol. *194*, 539–549.

Wolkow, T.D., and Enoch, T. (2002). Fission yeast Rad26 is a regulatory subunit of the Rad3 checkpoint kinase. Mol. Biol. Cell *13*, 480–492.

Woods, A., Sherwin, T., Sasse, R., MacRae, T.H., Baines, A.J., and Gull, K. (1989). Definition of individual components within the cytoskeleton of Trypanosoma brucei by a library of monoclonal antibodies. J. Cell Sci. *93*, 491–500.

Xu, Z., Vagnarelli, P., Ogawa, H., Samejima, K., and Earnshaw, W.C. (2010). Gradient of increasing Aurora B kinase activity is required for cells to execute mitosis. J. Biol. Chem. *285*, 40163–40170.

Yun, C.H., Mengwasser, K.E., Toms, A.V., Woo, M.S., Greulich, H., Wong, K.K., Meyerson, M., and Eck, M.J. (2008). The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc. Natl. Acad. Sci. USA *105*, 2070–2075.